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16. ABSTRACT

Three concurrent studies were conducted to determine the chronic effect of prespawning exposure to various concentrations of copper on fathead minnow reproduction. Copper was introduced into the three exposure systems to give 6-, 3-, and 0-months exposure prior to spawning. Prespawning exposure time had no significant effect on reproduction. Number of eggs produced per female decreased, however, with increase in copper concentrations. Egg production at copper concentrations of 37 $\mu g\ l^{-1}$ and higher was significantly lower ($P = 0.05$) than in the control, but at concentrations of 24 $\mu g\ l^{-1}$ and lower it was not different. The maximum acceptable toxicant concentration (MATC) was estimated to be 32 μg Cu l^{-1}, which is 0.07 of the 96 h LC50. This application factor for copper is similar to those found in other studies.

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EFFECT OF EXPOSURE TIME AND COPPER CONCENTRATION ON REPRODUCTION OF THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

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Abstract—Three concurrent studies were conducted to determine the chronic effect of prespawning exposure to various concentrations of copper on fathead minnow reproduction. Copper was introduced into the three exposure systems to give 6-, 3-, and 0-months exposure prior to spawning. Prespawning exposure time had no significant effect on reproduction. Number of eggs produced per female decreased, however, with increase in copper concentrations. Egg production at copper concentrations of 37 μg l^{-1} and higher was significantly lower ($P = 0.05$) than in the control, but at concentrations of 24 μg l^{-1} and lower it was not different. The maximum acceptable toxicant concentration (MATC) was estimated to be 32 μg Cu l^{-1}, which is 0.07 of the 96 h LC_{50}. This application factor for copper is similar to those found in other studies.

INTRODUCTION

The development of water quality criteria to protect aquatic life is complex for numerous reasons. A principal concern is the potential variability of biological response to stress as a result of variable exposure conditions. Rigidly controlled laboratory systems typically study continuous stress conditions. In reality, there can be an infinite variety of patterns of exposure to pollutants such as diurnal patterns due to plant operation, seasonal patterns due to rainfall, and patterns resulting from operations such as flow regulation, water-level control, temporary impoundment of liquid wastes, or intermittent waste discharge.

Since it is impossible to experimentally evaluate all possible exposure patterns, we selected a pattern for testing that consisted of constant exposure to the toxicant for different periods of time before spawning. Three previous chronic tests of copper toxicity to the fathead minnow (*Pimephales promelas*) indicated that the most sensitive life process is reproduction (Mount, 1968; Mount & Stephan, 1969; Brungs *et al.*, 1976). The fish survived and grew satisfactorily at copper concentrations higher than those affecting reproduction. The objective of our study was to evaluate the impact of copper on the reproductive success of the fathead minnow. In addition, these tests would develop information for evaluating the precision and repeatability of a chronic toxicity test and its associated application factor.

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METHODS

Exposure system

Three different exposure systems were used, each of which consisted of a proportional diluter (Mount & Brungs, 1967) that delivered 1 l of control water and 1 l of six copper concentrations per cycle to duplicate exposure chambers. A dilution factor of 0.6 was used to give nominal concentrations of 100, 60, 36, 22, 13, and 8 μg Cu l^{-1} and control. The concentrations were arranged randomly in each side-by-side row. The same random arrangement was used in all three exposure systems.

The glass exposure chambers were 60 × 30 × 30 cm high and contained 30 l of water. The flow rate to each chamber was about 200 l a day. Spawning substrates, consisting of six pieces of half-tile (Mount & Stephan, 1969), were placed in each chamber. Eggs were incubated in cups suspended from a rocker arm apparatus that oscillated two times per min (Mount, 1968).

Physical and chemical conditions

The dilution water, a mixture of spring-fed pond water from the Newtown Fish Farm and carbon-filtered demineralized Cincinnati tap water, was maintained at a hardness of 200 mg l^{-1} (as $CaCO_3$). Temperatures were recorded continuously in one chamber of each exposure system with 7 day indicating and recording thermographs. For the first 4 months the average temperature was 23 C with a maximum of 26 C. The mean temperature increased to 24 C by the end of May. During June, July, and August, when most of the spawning occurred, the mean temperature was 24.5 C. During September the mean temperature was reduced to 20 C by the end of the test.

Light was provided by cool white fluorescent ceiling fixtures controlled by two time-switches. Early in December the day length was reduced from 16 to 10 h. The day length was increased 1 h every month until 16 h were obtained in June, maintained for two months, and then decreased 1 h every month.

Routine analyses of the water from the exposure chambers were made in each set of duplicates every other week. Oxygen was measured in all seven chambers, hardness in the control chamber only, and pH, alkalinity, and

acidity in two chambers. In addition, hardness was measured in the dilution water every weekday. Procedures used were those described by the American Public Health Association (1965). The dilution water, similar to that used by Mount (1968), had a mean hardness of 202 ± 6.1 mg l^{-1} (as $CaCO_3$), and a mean acidity of 8 mg l^{-1}. The pH varied from 7.5 to 8.2 with a mode of 7.9. Dissolved oxygen had a mean concentration of 7.8 ± 0.74 mg l^{-1}.

The stock solution of copper was prepared by dissolving reagent-grade copper sulfate ($CuSO_4 \cdot 5H_2O$) in demineralized water. Copper, at similar concentrations, was added immediately in exposure system I, 3 months later in exposure system II, and immediately after the first spawning (6 months after the fish were added) in exposure system III. During copper dosing, water samples for copper analysis were removed daily from each chamber of one duplicate and composited for 7 days. Duplicate chambers of each system were sampled on alternate weeks. After acidification and extraction, these samples were analyzed for total copper by atomic absorption spectrophotometric procedures. Table 1 contains a summary of these copper analyses. Details of these methods have been described by Brungs *et al.* (1973). The mean extraction recovery of 128 spiked samples was 97% with a standard deviation of 2.9%. Measured copper concentrations reported in Table 1 were not corrected for recovery.

Biological system

All test fish were reared from eggs spawned in the laboratory. These eggs were collected and hatched, and the larvae were reared for about 4 weeks before they were introduced into the exposure chambers. On October 23, fifty-five larvae were assigned randomly to all 42 exposure chambers. Sufficient fish were retained for acute toxicity tests. Copper dosing began on November 4, in system I; on February 11, in system II; and on May 16, in system III.

The fish were fed a commercial dry trout food daily, and organisms in the water supply supplemented this diet. Excess food and waste products were removed from the exposure chambers as necessary. After 3 months the number of fish was randomly reduced to 20 or 21 per chamber. Later some males were removed to reduce territorial conflict.

During the spawning season all spawning substrates were examined in the early afternoon for eggs. After spawning activity was completed, newly deposited eggs were removed, examined for stage of development and counted. If more than one distinct stage of development was present, each stage was considered a spawning. Eggs, usually 50, were incubated and hatched in screen-bottomed cups. Where available about 10 spawnings were tested for hatchability. For the first 4 days of incubation, dead and live embryos were counted, and the live embryos were returned to the incubator. Hatchability was calculated as the percentage of larvae hatched after 7 days.

Acute toxicity

The acute toxicity of copper to fathead minnows from the same source as those used in the long-term test was determined with continuous-flow testing in the same dilution water. A diluter calibrated for a 0.75 dilution factor delivered test concentrations to duplicate exposure chambers containing 10 fish each. Two tests were conducted: one with 6-week-old fish (mean length 22 mm) and the other with 6-month-old fish (mean length 55 mm).

Statistical methods

Reproduction data from the long-term exposure were subjected to a two-way analysis of variance for factorial design with transformed values of $\sqrt{x} + \sqrt{x+1}$, where x is the mean number of eggs per female. The two controlled variables were length of time of prespawning exposure to copper and copper concentrations. The assumption was made that measured concentrations at the same nominal concentration were not different. The program used was "Analyses of Variance for Factorial Design" (Dixon, 1974). To test which concentrations were different from the control, a one-tailed Dunnett test (Steel & Torrie, 1960) was performed on the data.

In addition, the eggs-per-female data were analyzed according to the method (3–2.1.1) described by Natrella (1963). The assumptions were: (1) standard deviation varies linearly with the mean number of eggs per female; (2) the unknown mean number of eggs per female at which significance is obtained, when compared to the control mean, is based on five observations; and (3) at $P = 0.05$ and a two tailed t-test the $T_{0.975}$ ($d.f. = 4$) is ± 2.776. The unknown point at which a significant difference from the control occurred was then calculated by using the regression equation for standard deviation versus mean number of eggs per female.

The growth data were analyzed with analysis of variance from one-way design and Duncan's new multiple range test (Dixon, 1974).

The LC_{50} and EC_{50} (median effective concentration) values were determined by probit analysis, with a computer program based on Finney (1971).

RESULTS

Survival of fathead minnows was not affected by copper at any of the chronic exposure concentrations.

Table 1. Total copper concentrations in weekly composite samples from chronic toxicity tests

Nominal copper concentration (μg l^{-1})	Duplicate	System I (6-month prespawning exposure) Number of samples	System I Mean ± standard deviation (μg l^{-1})	System II (3-month prespawning exposure) Number of samples	System II Mean ± standard deviation (μg l^{-1})	System III (No prespawning exposure) Number of samples	System III Mean ± standard deviation (μg l^{-1})	Mean of all six chambers (μg l^{-1})
100	1	23	97 ± 9.3	17	96 ± 7.8	11	98 ± 11	98
100	2	23	101 ± 6.1	17	96 ± 10	10	101 ± 10	
60	1	24	57 ± 7.4	17	57 ± 4.1	11	61 ± 6.4	60
60	2	24	62 ± 4.5	17	58 ± 3.3	10	64 ± 5.8	
36	1	24	38 ± 4.8	17	36 ± 2.8	10	38 ± 4.7	37
36	2	24	38 ± 2.7	17	36 ± 1.7	10	38 ± 3.2	
22	1	24	25 ± 2.3	17	22 ± 1.6	10	22 ± 2.6	24
22	2	23	26 ± 3.1	17	24 ± 3.3	10	23 ± 2.3	
13	1	24	16 ± 2.0	17	15 ± 1.4	11	16 ± 1.9	16
13	2	23	17 ± 1.8	17	16 ± 1.4	10	16 ± 2.5	
8	1	24	12 ± 1.6	17	11 ± 1.2	11	10 ± 1.3	11
8	2	23	12 ± 1.5	17	11 ± 0.9	10	11 ± 1.2	
Control	1	24	4.9 ± 2.9	17	4.2 ± 1.1	10	3.5 ± 0.4	4.2
Control	2	24	4.2 ± 1.3	16	4.2 ± 1.5	10	4.3 ± 1.8	

Table 2. Spawning results and egg production—system I (6-month prespawning exposure to copper)

Mean measured copper concentration ($\mu g\ l^{-1}$)	Duplicate	Number of females	Mean length of females (mm)	Mean weight of females (g)	Number of males	Number of spawnings	Number of spawnings/ female	Total eggs produced	Number of eggs/ spawning	Number of eggs/ female
98	1	15	47	1.2	4	1	0.1	11	11	1
	2	16	52	1.5	5	5	0.3	463	93	29
60	1	12	49	1.9	5	34	2.8	2558	75	213
	2	11	55	2.0	8	8	0.7	708	88	64
37	1	13	58	2.5	5	45	3.5	5189	115	399
	2	12	56	2.1	7	73	6.1	7196	99	600
24	1	14	57	2.1	6	52	3.7	5937	114	424
	2	17	57	2.1	4	102	6.0	13,453	132	791
16	1	11	57	2.4	5	100	9.1	15,881	159	1444
	2	9	58	2.2	6	92	10.2	12,609	137	1401
11	1	7	56	2.2	5	65	9.3	12,361	190	1766
	2	8	57	2.2	4	60	7.5	10,052	168	1257
Control (4.2)	1	10	56	1.9	6	49	4.9	5700	116	570
	2	15	57	2.1	5	106	7.1	23,557	222	1570

Survival of the eggs that were spawned and incubated at all copper concentrations was not adversely affected by copper. In all three systems hatchability varied from 87%–97%, all within the normal range of variation found at this laboratory. Also, the length of time and adult fish were exposed to copper had no apparent influence on hatchability.

The 96 h LC_{50} values and 95% confidence limits were 490 (410–630) μg Cu l^{-1} for 6-week-old fry and 460 (390–540) μg Cu l^{-1} for the 6-month-old subadult fish.

Analysis of variance of the mean final length and weight of females (Tables 2, 3, and 4) indicated that the length and weight of the fish in the 3-month and no prespawning exposures were not significantly different ($P = 0.05$). However, the final length and weight of the females in the 6-month prespawning exposure were significantly different ($P = 0.05$). The Duncan test indicated that the final length of the females in the two high concentration was significantly shorter ($P = 0.05$) from that in the lower concentrations. However, the final weight of only the fish

Table 3. Spawning results and egg production—system II (3-month prespawning exposure to copper)

Mean measured copper concentration ($\mu g\ l^{-1}$)	Duplicate	Number of females	Mean length of females (mm)	Mean weight of females (g)	Number of males	Number of spawnings	Number of spawnings/ female	Total eggs produced	Number of eggs/ spawning	Number of eggs/ female
98	1	15	52	1.5	4	11	0.7	544	49	36
	2	11	55	1.9	9	2	0.2	70	35	6
60	1	14	56	2.0	6	10	0.7	715	72	51
	2	12	53	1.8	6	25	2.1	2854	114	238
37	1	11	56	2.1	10	26	2.4	2416	93	220
	2	13	55	1.9	7	56	4.3	6675	119	513
24	1	4	57	2.2	6	35	8.8	6255	179	1564
	2	7	59	2.6	2	31	4.4	6268	202	895
16	1	14	54	1.9	5	102	7.3	16,558	162	1183
	2	9	56	2.1	10	59	6.6	8281	140	920
11	1	8	55	2.1	6	30	3.8	3318	111	415
	2	17	57	2.3	3	75	4.4	11,199	149	659
Control (4.2)	1	13	55	1.9	7	106	8.2	18,664	176	1436
	2	13	55	2.2	7	33	2.5	5558	168	428

Table 4. Spawning results and egg production—system III (no prespawning exposure to copper)

Mean measured copper concentration ($\mu g\ l^{-1}$)	Duplicate	Number of females	Mean length of females (mm)	Mean weight of females (g)	Number of males	Number of spawnings	Number of spawnings/ female	Total eggs produced	Number of eggs/ spawning	Number of eggs/ female
98	1	15	55	1.8	4	7	0.5	866	124	58
	2	12	55	1.9	8	0	0	0	0	0
60	1	12	56	2.0	8	11	0.9	841	76	70
	2	16	55	2.1	5	37	2.3	2698	73	169
37	1	14	56	1.9	6	52	3.7	4992	96	356
	2	12	56	2.0	7	86	7.2	8798	102	733
24	1	11	55	2.0	8	29	2.6	3855	133	350
	2	13	55	2.1	6	83	6.4	12,884	155	991
16	1	15	56	2.2	5	84	5.6	10,796	129	720
	2	13	56	2.2	6	87	6.7	13,142	151	1011
11	1	11	57	2.2	9	54	4.9	6705	124	610
	2	12	55	2.0	6	96	8.0	12,329	128	1027
Control (4.2)	1	13	56	2.0	6	99	7.6	14,099	142	1084
	2	13	56	2.1	6	63	4.8	8930	142	687

in the high concentration was significantly less ($P = 0.05$) from that in the lower concentrations.

Spawning first occurred in a low copper concentration in system I on March 27 at a photoperiod of 13 h of light and a mean temperature of 22°C. During May, at a photoperiod of 14 h of light, fish started spawning in all systems. The first spawning in system III was on May 4, and copper was introduced on May 16, before the second spawning occurred. The last spawning was on September 22, at a photoperiod of 14 h and mean temperature of 22°C.

The effect of exposure time and concentration of copper on reproduction of the fathead minnow is summarized in Tables 2, 3, and 4. The total number of spawnings was reduced in the two high concentrations, and the mean number of eggs per spawning and the mean number of eggs per female were reduced in the three high concentrations. These data were analyzed statistically.

The statistical analysis indicated that the length of time of prespawning exposure to copper had no significant effect ($P = 0.05$) on reproduction (mean number of eggs per female). However, the effects of copper concentrations were highly significant ($P = 0.01$). The replicate exposure concentrations were combined, and the statistical analysis indicated that the mean number of eggs produced per female was reduced significantly ($P = 0.05$) at copper concentration of 37 $\mu g\ l^{-1}$ or higher; concentrations of 24 $\mu g\ l^{-1}$ and lower had no significant effect.

The regression equation (coefficient of correlation = 0.99) for the number of eggs per female and standard deviation was $Y = 0.162 + 0.504X$, where Y is standard deviation and X is the number of eggs per female. On the basis of increments of 25 eggs, 600 eggs per female was not significantly different ($P = 0.05$) from the control and 575 eggs per female was significantly different ($P = 0.05$). Regression analysis of the number of eggs per female versus copper concentration gave a parabolic curve (coefficient of correlation = 0.76) that was slightly better than a linear curve (coefficient of correlation = 0.74). When the parabolic equation was used, the copper concentration at which 600 eggs per female resulted was 32 $\mu g\ l^{-1}$. Thus 32 μg Cu l^{-1} was estimated to be the maximum acceptable toxicant concentration, and 34 μg Cu l^{-1} was estimated to be unsafe.

The EC_{50} for egg production (eggs per female) as compared to the control was estimated to be 40.4 μg Cu l^{-1} when regression analysis was used and 41.7 with 95% confidence limits of 39.2–44.5 μg Cu l^{-1} when probit analysis was used.

DISCUSSION

The results of this study clearly show that the length of time fathead minnows were exposed to copper before spawning had no significant influence on egg production. Apparently, the reduction in egg production due to copper is the result of exposure of the sexually mature fish only during spawning, i.e., the critical time of sublethal copper exposure is during the spawning season. Kihlstrom *et al.* (1971) reported a similar effect on the number of eggs produced by zebrafish. When spawning fish were exposed to various mercury (as phenylmercuric acetate) concentrations, spawning fell off rapidly, and the reduction was greater at higher concentrations. The effect of prespawning exposure was not studied.

The effect of copper concentration on egg production was highly significant ($P = 0.01$). This effect was a graded response with decreasing fecundity at higher concentrations. The relationship of egg production and copper concentration is best expressed by a parabolic curve, but this relationship is only slightly better than a linear relationship. Mount (1968) and Mount & Stephan (1969) reported an all-or-none response of fathead minnows, in which spawning at higher copper concentrations was completely blocked and lower concentrations had no effect. In contrast, we found a graded response, i.e., egg production decreased as copper concentration increased. This difference may be due to our use of more females. Mount (1968) exposed a total 8 females in duplicate exposure chambers; we exposed at least 63 females in our six replicate chambers.

Despite this different response, our results are in excellent agreement with those reported by Mount (1968). In both chronic studies the fathead minnow was exposed to copper in a similarly hard water (200 mg l^{-1} as $CaCO_3$). Mount (1968) found that a concentration of 33 μg Cu l^{-1} completely blocked spawning; we found that a concentration of 37 μg Cu l^{-1} significantly reduced egg production ($P = 0.05$). He found that a concentration of 14.5 μg Cu l^{-1} had no effect on egg production; we found that a concentration of 11 μg Cu l^{-1} was not significantly different ($P = 0.05$) from the controls. Our intermediate concentration of 24 μg Cu l^{-1} also was not significantly different ($P = 0.05$) from the control.

Egg production of the fish in 16 μg Cu l^{-1} was the highest for all of the exposure chambers. The mean number of eggs per female was 122% of that in the control; however, the difference was not significant ($P = 0.05$). Also the variation in the number of eggs per female was less than in other concentrations and controls. The data for egg production at 16 μg Cu l^{-1} were not used in the statistical estimate of the MATC or in the probit analysis of the EC_{50}.

Mount (1968) indicated that growth of the fathead minnow could not be used to estimate the MATC of copper because the effect on reproduction was more sensitive. He found that for the first 8 weeks growth rate was related to copper concentration; however, after 16 weeks, there was no effect on growth rate except at the high concentration of 95 $\mu g\ l^{-1}$. Our study and those of Mount (1968), Mount & Stephan (1969), and Brungs *et al.* (1976) indicate that in the fathead minnow, growth is a less sensitive indicator of the effect of copper than reproduction.

The applicability of a statistically significant effect found in a toxicity test to biological significance is a most difficult evaluation. A statistically significant effect may not be deleterious to the individual fish or the population. With improvement of methodology and design of chronic tests, effects can be found at lower and lower concentrations to be statistically different from the control. In a natural environment there is much natural variation in the reproductive success of a population. Waller *et al.* (1971) reported on a program designed to simulate the effects of reduction in egg production on population size of fathead minnows. On the basis of their model they concluded that a 50% reduction in egg production would not result in the extinction of a fathead minnow population, but if the reduction in egg production is increased from 50% to 79%, the population would become extinct.

The MATC value of 32 μg Cu l^{-1} was estimated to cause a 30% reduction in egg production. The application factor (Mount & Stephan, 1967) calculated from this MATC value and a continuous-flow 96 h LC_{50} value of 460 μg Cu l^{-1} is 0.07. This application factor lies within the range of 0.03–0.07 reported by Mount (1968).

McKim & Benoit (1974) reported that short-term exposure of the early life history stage of the brook trout to copper would provide "no effect" concentrations similar to that of the chronic toxicity test. Our results indicate that short-term exposure during spawning would also give "no effect" concentrations. Although chronic exposure of brook trout and fathead minnows to copper affects production at different life history stages, the "no effect" concentration of chronic exposure is dependent upon the short-term exposure during the sensitive life history stage. However, it is critical that the sensitive life history stage be exposed to predict chronic toxicity.

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